Evidence for the archaebacterial-type conformation about the bond between the β-ionone ring and the polyene chain of the chromophore retinal in chlamyrhodopsin

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Abstract Previous studies using retinal analogs (Wada, A., Sakai, M., Kinumi, T., Tsujimoto, K., Yamauchi, M. and Ito, M. (1994) J. Org. Chem. 59, 6992–6997; Wada, A., Sakai, M., Imamoto, Y., Shichida., Y., Yoshizawa, T. and Ito, M. (1993) Chem. Pharm. Bull. 41, 793–795) revealed that both retinochrome and visual pigments share a common chromophoric conformation in which the ring region of retinal is twisted ca. 50° with respect to the plane of the polyene chain, suggesting a highly conserved 6-s-*cis* conformation throughout rhodopsin-like pigments in animals. By contrast, 6-s-*trans* conformation was commonly observed or suggested in archaebacterial rhodopsins examined thus far. Here we have reconstituted in vivo both the photoreceptor for photobehavioral responses of the unicellular alga *Chlamydomonas reinhardtii* and the second archaeal sensory photoreceptor phoborhodopsin from a series of retinal analogs. Results exclusively point to the conclusion that in both photoreceptors retinal has the coplanar 6-s-*trans* conformation, though recent molecular cloning studies revealed no homology between *Chlamydomonas* photoreceptor (chlamyrhodopsin) and archaeal rhodopsins.

Key words: Retinal analog; Phoborhodopsin; Bacteriorhodopsin; Phototaxis; *Chlamydomonas reinhardtii*; *Halobacterium salinarum*

1. Introduction

Archaebacterial rhodopsins and visual pigments in animals share a seven transmembrane segment topology, accommodating their common chromophore retinal, which is covalently linked to a lysine residue located roughly in the middle of their seventh α-helix segment. Despite such similarities, however, their functional chromophore is distinguishable in terms of stereochemistry. Signal transduction cascade of animal visual system is initiated by 11-*cis*→all-*trans* photoisomerization, whereas in archaebacterial rhodopsins all-*trans*→13-*cis* photoisomerization triggers their diverse biological functions [1,2]. So far, among rhodopsin-type photoreceptor families in the animal kingdom, the only exception regarding the configuration of the functional chromophore is retinochrome [3], which reisomerizes retinal from all-*trans* to 11-*cis* form in visual cells of mollusks.

In addition, these two rhodopsin-type photoreceptor families do not share a common chromophoric conformation about a single bond joining the β-ionone ring region to the conjugated polyene chain. The retinylidene chromophore of visual pigments has twisted s-*cis* conformation about the C6-C7 bond [4]. However, in bacteriorhodopsin, the conformation is the coplanar s-*trans* [5]. Reconstitution experiments using artificial 'locked' retinal analogs in which the ring and the polyene chain was connected through an extra 6-membered ring also suggested the 6-s-*trans* conformation for the chromophore of other two archaebacterial rhodopsins, i.e. halorhodopsin and sensory rhodopsin I [6]. The use of related analogs, having an extra 7- and 8-membered ring in which the connections were conducted through an ethylene and a propylene group, respectively, has revealed that the torsional angle about the C6-C7 bond is ca. 50° in both bovine rhodopsin [7] and retinochrome [8]. As far as we know, reconstitution study with these coplanar or twisted bicyclic analogs has not yet been reported on phoborhodopsin (PR, also referred to as sensory rhodopsin II; the fourth archaebacterial rhodopsin [9]) except for an analog having a naphthyl group instead of the β-ionone region [10].

The unicellular green alga *Chlamydomonas reinhardtii* (*C. reinhardtii*) also uses retinal as the chromophore for its photosensory function [11–14]. Recently the gene encoding putative apoprotein of the photoreceptor was cloned, and the name chlamyrhodopsin has been proposed [15]. Unlike other eukaryotic rhodopsins, the *Chlamydomonas* photoreceptor requires all-*trans* to 13-*cis* photoisomerization for triggering behavioral responses [12–14,16]. A preliminary test using 6-membered, 6-s-*cis* and 6-s-*trans* locked analogs suggested an archaebacterial-type conformation for its chromophore retinal [14,16]. However, chlamyrhodopsin exhibited no apparent homology to archaebacterial rhodopsins in its protein primary sequence [15], but a slight homology to invertebrate rhodopsin has been suggested [15,17]. Considering the facts that less experimental data have been accumulated regarding the issue of ring-polyene chain conformation than that of the configuration/photoisomerization event of the chromophore, and that the characteristic dihedral angle of ca. 50° is essentially conserved widely among rhodopsin-type photoreceptors in animals, we thought it necessary to confirm whether or not chlamyrhodopsin and other eukaryotic rhodopsins share a conserved feature other than the reported slight homology. Here we report the results of a comprehensive reconstitution study on the conformation of the C6-C7 bond of the chromophore in both the *Chlamydomonas* photoreceptor and archaebacterial rhodopsins including PR (Scheme 1).

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2. Materials and methods

C. reinhardtii strains FN68 and CC2359 were grown as reported previously [12]. All-*trans* bicyclic retinal analogs were synthesized as reported [18,19]. The analogs and all-*trans*-retinal (Sigma) were used as ethanolic solutions.

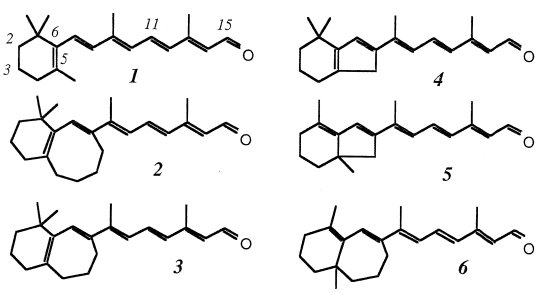
Chlamydomonas cells were kept in the dark for 4 h after addition of all-*trans* retinal or analogs. Photobehavioral responses of the cells were assayed on an inverted microscope (TMD, Nikon, Tokyo, Japan) equipped with an infrared sensitive CCD camera [12] connected to a commercially available video motion analyzer system (Motion Analysis Corp., Santa Rosa, CA, USA). Both phototactic orientation and photophobic responses were determined using newly developed software; however, because complex factors affect the direction of phototaxis [20], only photophobic response was used as the measure of the activity of photoreceptors reconstituted from native and analog chromophores.

Reconstitution of archaebacterial rhodopsins in apo-membrane preparations from *Halobacterium salinarum* (*H. salinarum*) was monitored using SLM-Aminco DW-2000 spectrophotometer [10]. Photophobic response of *H. salinarum* was measured 6 h after addition of retinal and analogs to strain Flx3b1 cell suspensions, on an epi-fluorescence microscope (X2F, Nikon) connected to the motion analyzer system. Since absorption maxima of reconstituted PR analogs in intact Flx3b1 membrane distributed within a relatively narrow spectral range (470∼495 nm), a 480-nm band-pass filter with a half bandwidth of 14 nm was used throughout the experiments for actinic irradiation (5×10^{14} photons/mm^2). Other experimental details were essentially the same as described elsewhere [21].

3. Results and discussion

Fig. 1 shows the ring portions of all-*trans* retinal (**1**) and analogs whose conformation energies were minimized by molecular mechanics calculation using MM2 [22]. In solution, the native retinal is known to have 6-s-*cis* conformation and the C1-C6-C7-C8 torsional angle is almost identical to that of retinal analogs **2** and **3** [7]. As had been confirmed by reconstitution experiments and circular dichroism measurements, analog **3** represents the most probable conformation about the 6-7 bond of native retinal when accommodated either in bovine rhodopsin or in squid retinochrome [7,8]. On the other hand, the conformation of retinal in bacteriorhodopsin is 6-s-*trans* [5] and thus mimicked by both 6 membered 6-s-*trans* locked retinal [6] and the 5-membered analog **5**. Our MM2 calculation gave −162° and −167° for the C5-C6-C7-C8 torsional angles of analog **5** and the 6-membered 6-s-*trans* locked retinal, respectively.

Results of reconstitution experiments using apo-membrane suspensions each containing only one species of archaebacterial rhodopsins [21] are shown in Table 1. The apoprotein of PR accommodates **3** and **5** almost equally well, and less affinity to analog **4** is clearly seen from both slow kinetics in pigment regeneration in vitro and a higher concentration re-



Scheme 1. Retinal and analogs used in this research.

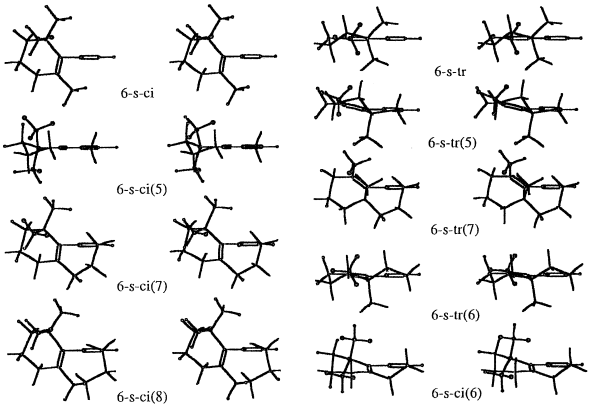


Fig. 1. Stereoview of the ring portions of retinal and bicyclic analogs from the direction of the C8-H bond. Conformational energy was minimized by MM2 for each complete molecule but only regions proximal to the ionone ring are shown. **6-s-ci** and **6-s-tr** indicate 6-s-*cis* and 6-s-*trans* conformers of all-*trans* retinal, respectively. **6-s-ci(5)**, **6-s-ci(6)**, **6-s-ci(7)** or **6-s-ci(8)** indicates the analog having a locked 6-s-*cis* conformation with an extra 5-, 6-, 7- or 8-membered ring, respectively. **6-s-tr(5)**, **6-s-tr(6)** or **6-s-tr(7)** indicate 6-s-*trans* locked analog containing an extra 5-, 6- or 7-membered ring, respectively.

quired for in vivo restoration of photophobic response. This discrimination between analogs **4** and **5** provides a support for the previously hypothesized view that appearance of vibrational fine structure in the absorption spectrum of PR is due to coplanar 6-s-*trans* structure of the chromophore [10]. The absorption maximum of PR analog reconstituted from **5** was 495 nm, which is closer to that of PR (487 nm) than that of the one reconstituted from **3** (470 nm). Opsin shift values calculated as described [18] revealed that only 120 cm^{-1} is attained when analog **5** is incorporated into the apoprotein of PR, supporting the previous view that most of the opsin shift of native chromophore in PR (2100 cm^{-1}) is due to ring-chain coplanarization [10]. By contrast, the opsin shift of analog **3** was 950 cm^{-1}, presumably as a consequence of the expected slight flexibility of the 7-membered ring, suggesting a more coplanar conformation along the C6-C7 bond at the binding site than in the model Schiff base compound in solution. From these data, we concluded the 6-s-*trans* conformation for the retinylidene chromophore of PR.

On the other hand, both bacterioopsin (Bop) and sensory opsin I (Sop-I) accommodated analogs **4** and **5** almost equally well, suggesting that most of archaebacterial rhodopsins (except PR) distinguish the dihedral angle of the C6-C7 bond. This indicates that the difference in affinities to the coplanar bicyclic analogs **4** and **5** may not be used as a single criterion to test whether or not a retinylidene pigment has an archaebacterial-type chromophore binding site. However, analog **3**, which showed relatively slow regeneration kinetics with Bop, was hardly incorporated into Sop-I, confirming the coplanar 6-s-*trans* conformation in sensory rhodopsin I.

From these results, we suspect that there are two critical parameters for the discrimination between 6-s-*trans* and 6-s-*cis* conformers: one is steric hindrance due to the ring methyl groups (1-methyl, and/or 5-methyl), and the other the coplanarity of the ring region with respect to the polyene chain. We think the former is more essential in phoborhodopsin, because it discriminates 5-membered 6-s-*trans* and *cis* analogs (analogs **4** and **5**), differing in this respect from bR and sensory rho-

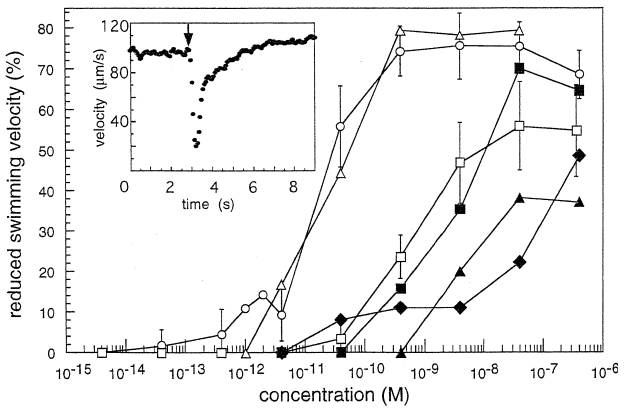


Fig. 2. Dose-response curves for photophobic response of *C. reinhardtii* strain CC2359 cells reconstituted from retinal and analogs. Reduced swimming velocity of cells (see inset) shortly after onset of the light stimulus (470 ± 15 nm, 2.3×10^{14} photons/mm^2) was averaged over a cell population, normalized by an average velocity of the cells under unstimulated condition, and plotted. Open and closed symbols indicate analogs were locked in 6-s-*trans* and 6-s-*cis* conformations, respectively, except for (○), which represents cells reconstituted from native retinal. Analogs containing an extra 5-, 7- and 8-membered ring are indicated by (△, ▲), (□, ■) and (◆), respectively. Bars represent standard errors ($n = 3$). Inset: Typical time course of a change in swimming velocity of cells averaged over >200 tracks in a cell population. The arrow indicates the timing of onset of actinic light.

dopsin I, and the latter is most evidently seen in sensory rhodopsin I, which showed rather ambiguous discrimination between 6-membered 6-s-*trans* and -*cis* analogs in the previous report by Baselt et al. [6]. Although both **3** and **5** were incorporated into the apoprotein of PR, a small but significant difference between the half maximal concentrations for restoring photophobic response could be seen (Table 1).

As a consequence, we concluded that the reconstitution experiment using analog **3** as well as those with coplanar bicyclic analogs is important for the characterization of the retinal binding site of chlamyrhodopsin, since previous reports from Spudich's group only briefly described that 6-membered 6-s-*trans* locked analog restored phototaxis and photophobic response to 50% and 90% in magnitude, respectively, at concentrations where 6-membered 6-s-*cis* locked analog regenerated no photobehavioral response [14,16].

Fig. 2 shows the photophobic response of *Chlamydomonas* cells reconstituted with retinal and analogs. At present, consensus has been obtained that both phototactic and photophobic responses are mediated by a single retinylidene photoreceptor [12,23,24]. Analog **3** required two orders of magnitude higher concentration to restore photophobic response compared with native retinal and analog **5** (Fig. 2). Analog **5** exhibited almost identical dose-response relationship as did native retinal. These data strongly suggested that chlamyrhodopsin has an archaebacterial-type retinal binding site.

Among the 6-s-*cis* locked retinals examined, the most effective one in restoring the response is **3**. Because of this and the fact that *Chlamydomonas* photoreceptor distinguishes analog **5** from **4** in contrast to Bop and Sop-I, one might speculate that chlamyrhodopsin has a retinal binding site phylogenetically related to PR. Resemblance between the absorption spectrum of PR and the earlier microspectrophotometric recording of chlamyrhodopsin [25] may support this view. However, not even a slight homology either to PR or to other archaebacterial rhodopsins could be observed in the amino acid sequence of chlamyrhodopsin [15,26]. Furthermore, a signal transduction sequence analogous to mammalian G protein-coupled processes has been suggested quite recently for phototaxis of a flagellated alga closely related to *Chlamydomonas* [27]. At present, chlamyrhodopsin and related species (e.g. volvoxopsin [17]) seem to constitute a unique retinylidene protein family among those identified thus far. To reconcile our data with those findings, we currently interpret the possible phylogenetic relationship as follows. First, we assume that at the early stages of evolution of photoreceptors, where microorganisms had to extend their photosensory functions to a longer wavelength region, there must be a variety of candidates. Regardless of their chromophoric group (not necessarily retinal), it is likely that photoreceptors having all-*trans* conformation/configuration were most popular at this stage; because for extending their absorption maxima toward a longer wavelength region, it is advantageous for their chromophore(s) to have a double-bond system relatively well-conjugated. If such geometry has been conserved during evolution, photoreceptors having 6-s-*trans* conformer would constitute a majority among those which utilize retinal as a chromophore, despite a drawback that the conformational change from 6-s-*cis* to 6-s-*trans* is rate-limiting in the chromophore binding processes [28,29]. Thus, from our present view, it is natural that phylogenetically distinct retinylidene photoreceptor species in microorganisms share the same 6-s-*trans* conformation of their chromophore.

In conclusion, we have shown that the chromophore of both photoreceptors, PR from *H. salinarum* and the *Chlamydomonas* phototaxis receptor, share the coplanar 6-s-*trans* conformation with that of other archaebacterial rhodopsins.

Table 1
Reconstitution of archaebacterial rhodopsins from retinal analogs

Retinal analogs	Tortional angles (deg)[a]	Reconstitution		
		Bacteriorhodopsin[b]	Sensory rhodopsin I[b]	Phoborhodopsin[b,c]
2	61	Slow	n.d.[d]	n.d.[d]
3	50	Slow	No pigment	Slow (8×10^{-10} M)
4	−179	Fast	Fast	Very slow[e] (10^{-8} M)
5	−162	Fast	Fast	Slow (5×10^{-10} M)
6	−140	Slow	Slow	Slow

[a]C5-C6-C7-C8 dihedral angles (counterclockwise, positive); the numbering of the atom is according to the retinal molecule.
[b]Designated as slow if more than half of the opsin remained unreconstituted 2 h after addition of each analog chromophore.
[c]Half maximal concentration required to restore photophobic response in a cell suspension of *H. salinarum* strain Flx3b1 (in parentheses).
[d]Not determined.
[e]Only partial reconstitution was observed even after 24 h.

However, in order to clarify phylogenetic position of the unique photoreceptor chlamyrhodopsin, it is important in future studies to look for other unidentified photoreceptor species possibly present in various (micro)organisms.

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